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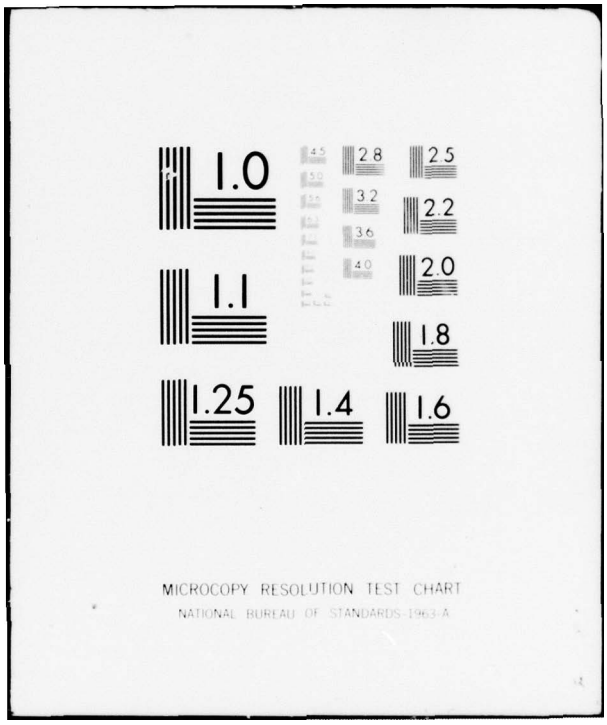
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IDENTIFICATION OF MICROORGANISMS BY THEIR PURINE AND PYRIMIDINE CONTENT

by

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April 1977

STANFORD RESEARCH INSTITUTE
Menlo Park, California

Contract DAAA15-73-C-0132

DEPARTMENT OF THE ARMY
US Army Armament Research and Development Command
Chemical Systems Laboratory
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Methods have been studied for identifying microorganisms by means of their nucleic acid base ratio patterns. Field ionization mass spectrometry techniques are employed which potentially allow the analysis of underrivatized free bases from 10^4 bacteria with 1% precision. Techniques are described for isolating DNA and RNA from small quantities of bacteria in 1-1/2 hours. A hydrolysis technique using liquid HF was developed that quantitatively releases the bases from DNA in 8 minutes at 25°C or from RNA in 16 minutes at 35°C. No destruction of purines

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is observed with this technique. Small quantities of bacteria can be analyzed by using E. coli labeled with ^{15}N as a "carrier" during the isolation and hydrolysis procedures.

The ^{15}N-labeled bases from the carrier also serve as internal standards in the mass spectrometric analysis. Thus the known ratios of the labeled bases from the added E. coli can be compared to the base ratios of the unlabeled bases from an unknown organism to compensate for differences in ionization efficiencies within the multicomponent mixture.

PREFACE

The research described in this report was authorized under Project/Task 1L161102A71A-01, Basic Life Sciences Research in Support of Biological Defense Material. It was performed at Stanford Research Institute by the Mass Spectrometry Research Center under Contract No. DAAA15-73-C-0132. The technical studies in this contract were completed in June 1976.

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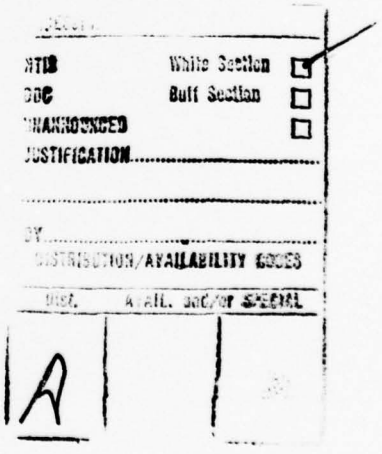

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I. INTRODUCTION

The goal of this project has been to collect and analyze a small sample of biological material to detect the presence of viral or bacterial pathogens by the purine and pyrimidine content (i.e., nitrogen bases) of their nucleic acids, with the further objective of identifying the pathogen by pattern recognition of the base compositions of both DNA and RNA in the observed species.[1] Our techniques, which can determine the relative concentrations of the bases with better than 1% precision, might allow species identification on the basis of minor differences in the relative concentrations of the major bases. The methylated bases have been shown to play a role in making DNA or RNA species-specific[2] and, thus, are a source of additional identification information.

Field ionization mass spectrometry (FIMS) is the only analytical technique that combines both the sensitivity and the specificity required for the identification of airborne pathogens by their purine-pyrimidine content. This technique, which produces predominantly molecular ions, is ideally suited for this analysis because it allows quantitative analysis of many components simultaneously.

Methodologically, the procedure for detection and identification of pathogens consists of the following steps:

(a) Collection of particulate matter from air carrying potential pathogens.

(b) Separation of microorganisms and viruses from other biological particles.

(c) Extraction of the nucleic acids from the microbiological sample.

(d) Hydrolysis of the nucleic acids to give the free purines and pyrimidines.

(e) Quantitative analysis of the purines and pyrimidines.

A three-phase research program was conducted to accomplish these objectives over a three-year period.

During phase I, we established the feasibility of FIMS for the detection of pathogens and developed the basic methodology for step (e). We also explored various techniques to accomplish step (d), which was identified as a limiting step in the total process, as the available methods for nucleic acid hydrolysis were slow and nonquantitative.

During phase II, we developed methods to accomplish step (d) in 8 minutes for DNA and 16 minutes for RNA. We continued to improve the analytical instrumentation, and the ability to quantitatively hydrolyze DNA allowed us to identify various problem areas in step (e) that require further development in instrumentation or methodology. Our present instrumental sensitivity, as established by quantitative measurements of purine and pyrimidine samples of known concentration, allows determination of the purine-pyrimidine composition of 10^4 bacteria or 10^6 virus particles with 1% precision.

Steps (a) and (b) can be accomplished within the present state of the art to allow the entire method to be evaluated at the sensitivity of at least 50 aerosol particles per liter of air.

During phase III, we developed a rapid and efficient nucleic acid extraction method (step (c)). Also we used ^{15}N-labeled E. coli as a carrier and a multicomponent-labeled internal standard to improve the analysis in step (e).

II RESEARCH PROGRAM

A. Mass Spectrometry

Conventional mass spectrometry using electron impact for ionization can have a fairly high ionization efficiency (of the order of 1 in 10^4), but the ionization produces extensive fragmentation. Moreover, the extensive background produced by the complex fragmentation reactions overshadows most of the mass numbers of importance. The only type of mass spectrometry amenable to our problem of simultaneous determination of 5 to 10 purines and pyrimidines is nonfragmenting field ionization with a sufficiently high ionization efficiency.

1. Multipoint Field Ionization Source

The multipoint field ionization source recently developed at SRI has considerably improved the overall sensitivity attainable with field ionization, making this technique a releiable analytical tool.[3] The entire source construction, showing points, grid, heater, and insulators is illustrated in figure 1. This source allows sustained operation at high temperatures to minimize sample memory and background interferences. Silanization of the entire source structure has further reduced sample memory because of adsorption within the source, as well as reducing pyrolytic destruction of the thermally labile constituents such as guanine. Sources with 50-60 μm point-to-counterelectrode spacing have been operated at a low enough initial ion energy (less than 1000 V) to allow reliable interfacing with a quadrupole mass spectrometer. Here we encounter losses in ion transmission resulting from deceleration of the beam to the low energies (10-20 eV) required for the quadrupole. With mass analyzers such as the magnetic sector or ion velocity filter, which operate at higher beam energies, a better ion transport efficiency is obtained.

During phase III, we developed a rapid-temperature-cycle multipoint field ionization source, shown in figure 2. This source has a shortened sample path length and an overall lower thermal mass than the previous source. Hence, the source can be operated at one temperature (250°C), baked out at a higher temperature between runs (300°C), then recycled to the lower operating temperature in a short time. Both new features reduce the memory from one sample to the next.

Solid samples are introduced by use of a quartz probe with a 2 x 20 mm sample cavity in the end. In this system, the vaporized sample is transmitted with high efficiency to the ionizing region. The probe temperature can be controlled independently of the source temperature by a gas or liquid coolant inlet tube and a Nichrome ribbon heater coiled around the sample cavity. By the proper choice of coolant and heater power, the probe temperature can be controlled from liquid nitrogen temperature to 500°C.

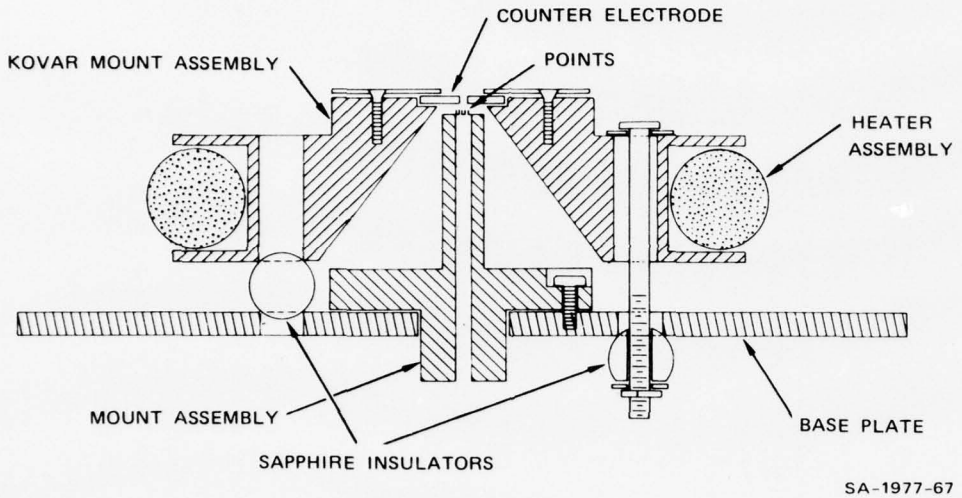


Figure 1. High Temperature Field Ionizer with Sapphire Insulators

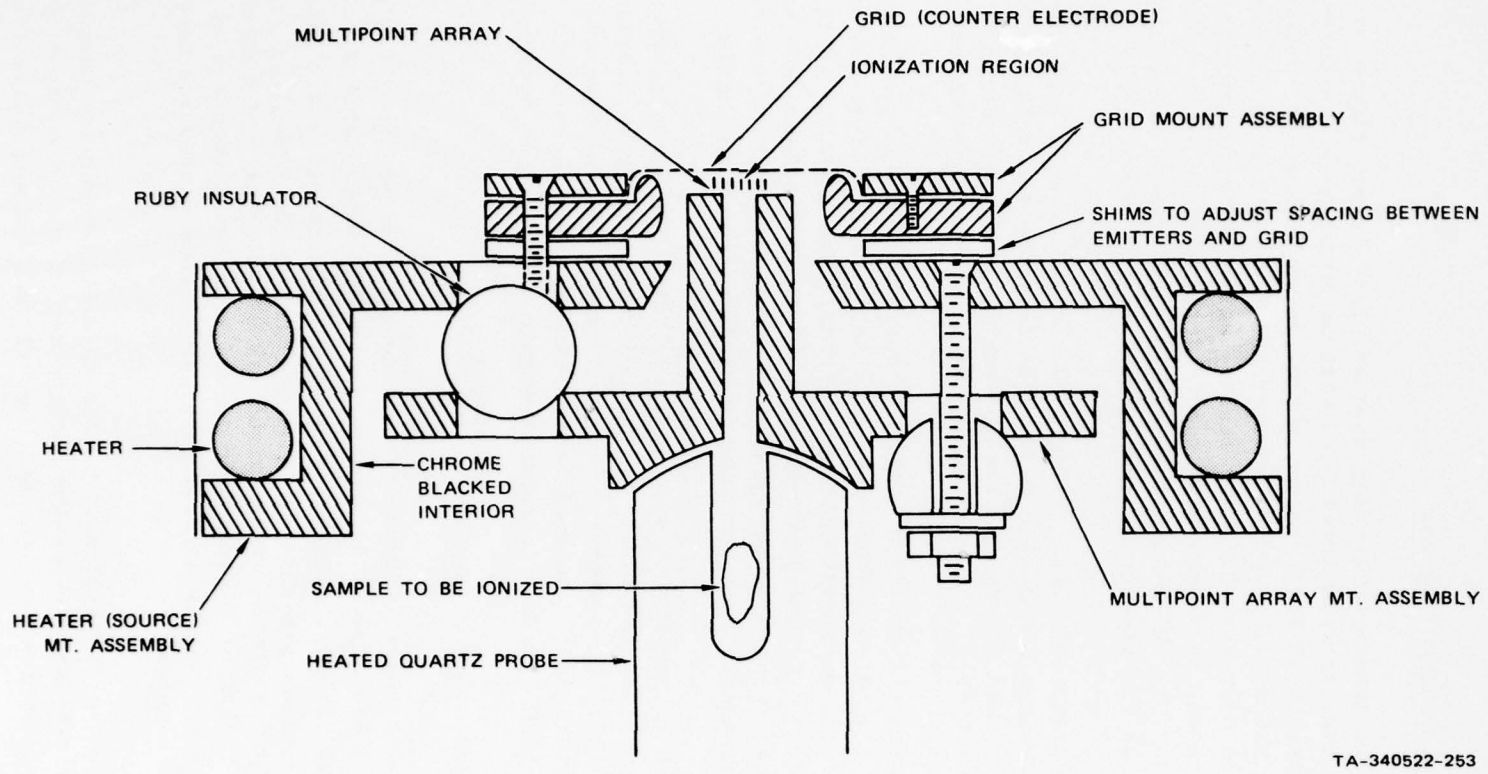


Figure 2. Standard Multipoint Field Ionizer Assembly

2. Mass Spectrometers

The mass spectrometer used in phase I (see figure 3) consists of a multipoint ionizer interfaced with an Extranuclear quadrupole Model 270-9 equipped with ELFS system, which provides increased resolution and ion transmission. The entire ionizer and lens system is surrounded by an oven capable of maintaining the source region at temperatures up to 350°C. During phase II, we used a mass spectrometer with an ion velocity filter as mass selector, shown in figure 4. This instrument has a higher transmittance than the quadrupole analyzer.

During phase III, we used 60° and 90° sector magnet mass analyzers. These instruments are easier to operate than the ion velocity filter mass spectrometer, and the resolution is greatly improved. Figure 5 illustrates the configuration of the new sector magnet mass spectrometers.

With all the mass analyzers currently in use, an integrated profile of a multicomponent mixture is obtained by adding successively scanned spectra on a multichannel analyzer. The electron multiplier detector is used in the pulse-counting mode. The pulses from an amplifier discriminator are stored in the multichannel analyzer to provide an integrated mass-analyzed ion current profile from each sample.

For the sector magnet spectrometers, the mass range covered by the nucleic acid bases can easily be scanned repetitively by programming the magnetic field. With magnetic scanning, the more sensitive magnetic sector instruments can be scanned rapidly enough to accurately integrate the evaporation of a multicomponent sample in less than 5 minutes.

3. Efficiency with Nitrogen Bases

Our present instrumentation and solid sample handling systems can analyze a hydrolyzed DNA or RNA sample, yielding simple spectra of molecular and $(M + H)^+$ ions for each nitrogen base present. Examples of typical spectra are shown in figures 6, 7 and 8. Chemical cleanup of the dried hydrolysate is not required, as no interfering background ions in the mass region of interest are formed from the components of the hydrolysis residue or from proteins carried through the same procedures used for nucleic acid samples.

We have examined most of the naturally occurring purine and pyrimidine bases, as well as the nucleosides of the five major bases using FIMS. Various derivatives (methyl, acetyl, and trimethylsilyl) of these

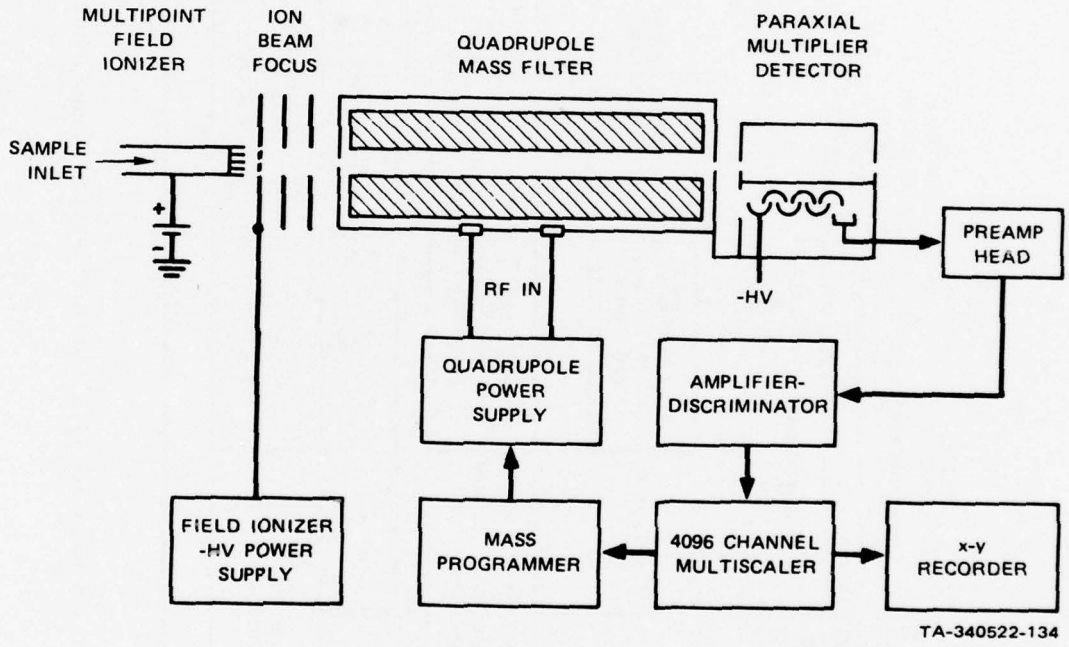


Figure 3. Schematic of Field Ionization Source, Quadrupole System

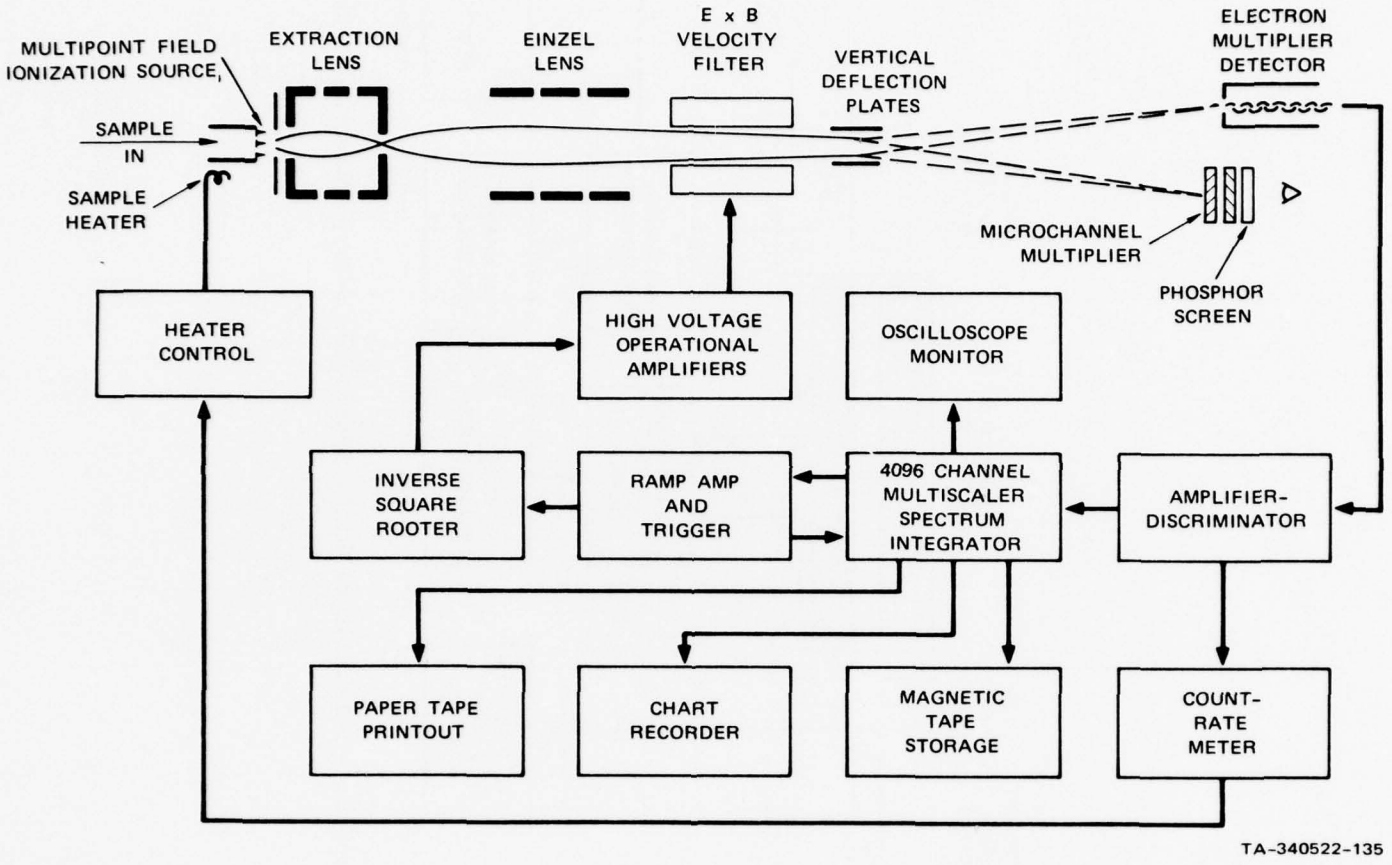


Figure 4. Schematic of Field Ionization Fingerprint Apparatus

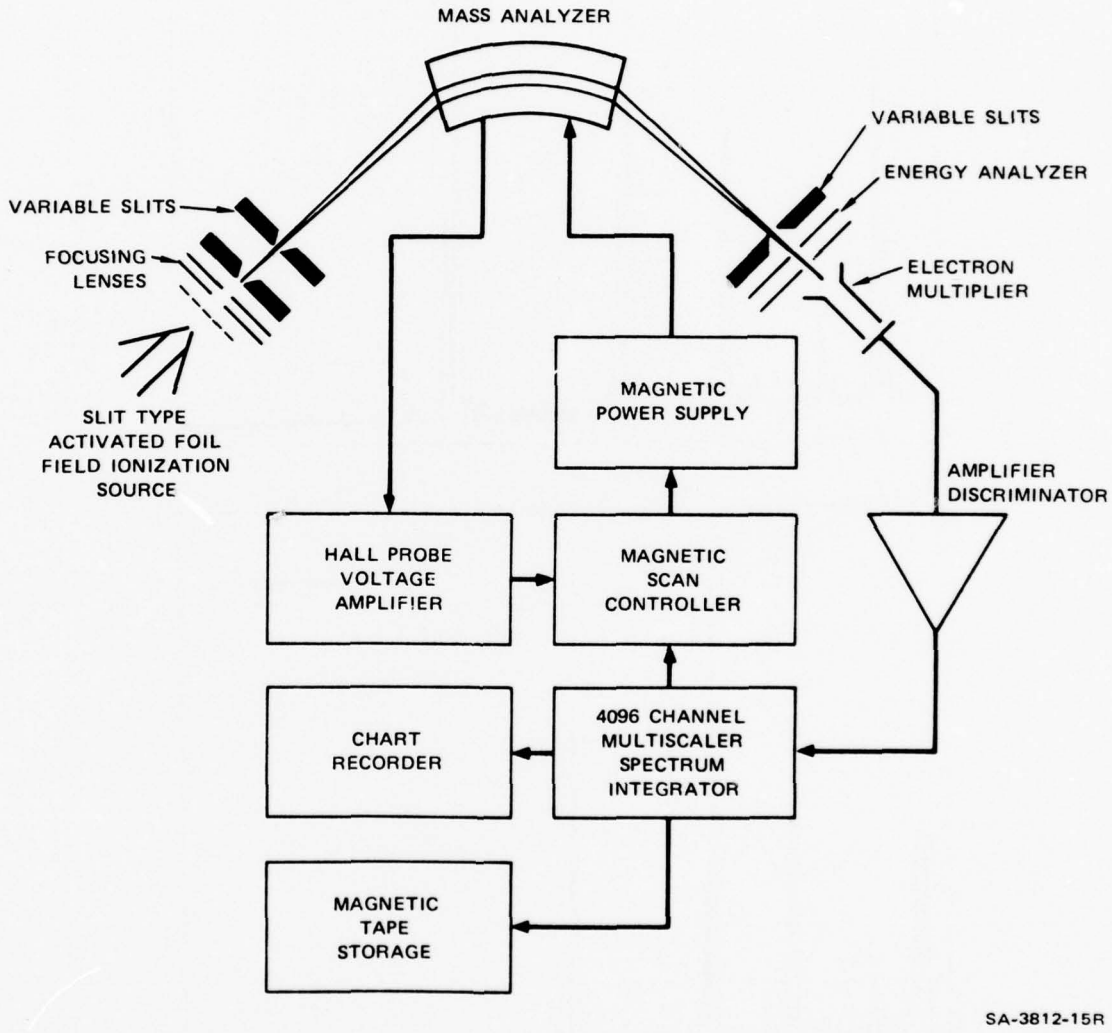


Figure 5. Schematic of Field Ionization Sector Magnet Mass Spectrometer

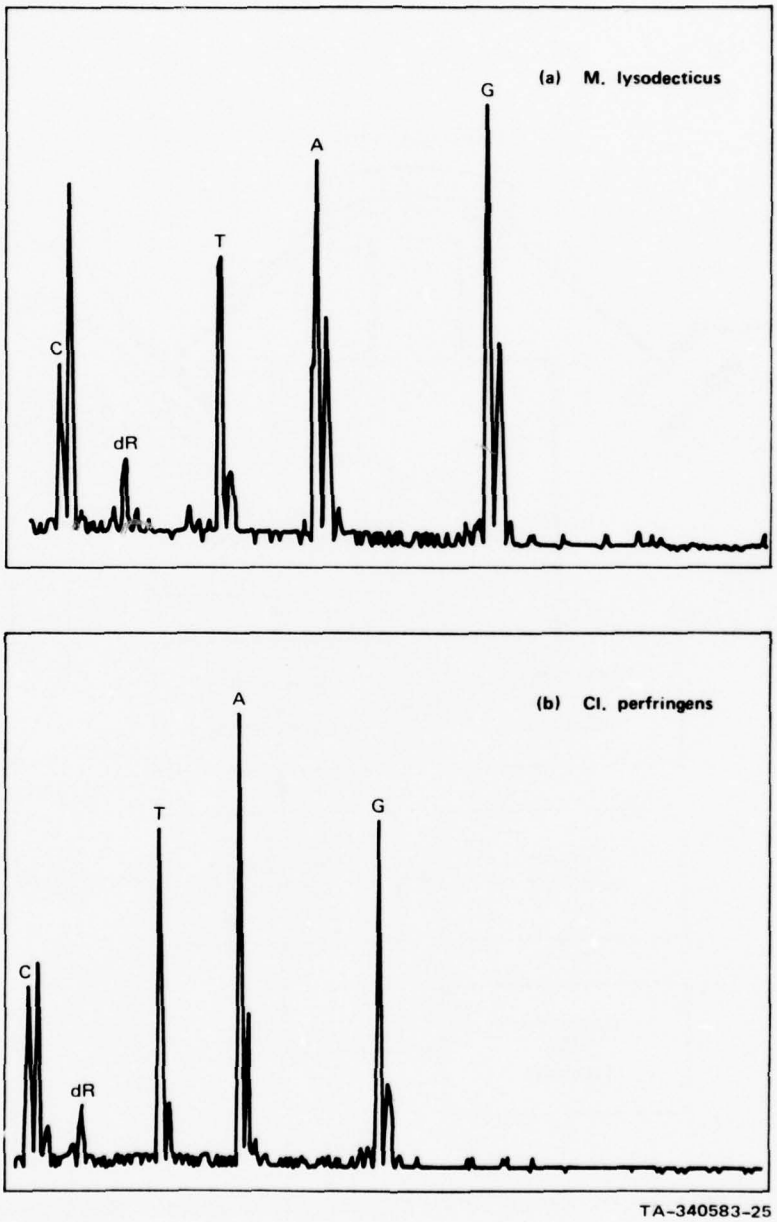


Figure 6. Fingerprint Pattern from DNA

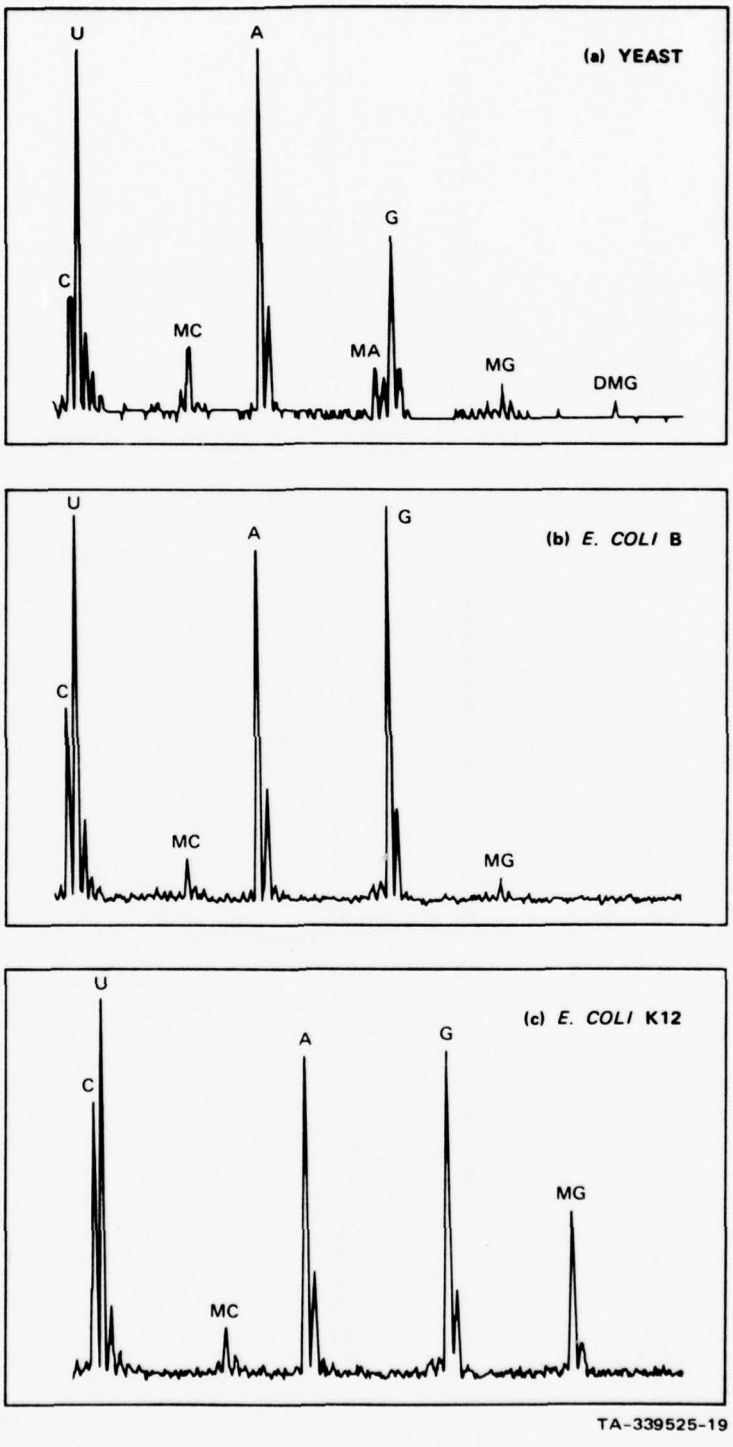


Figure 7. "Fingerprint" Patterns from t-RNA

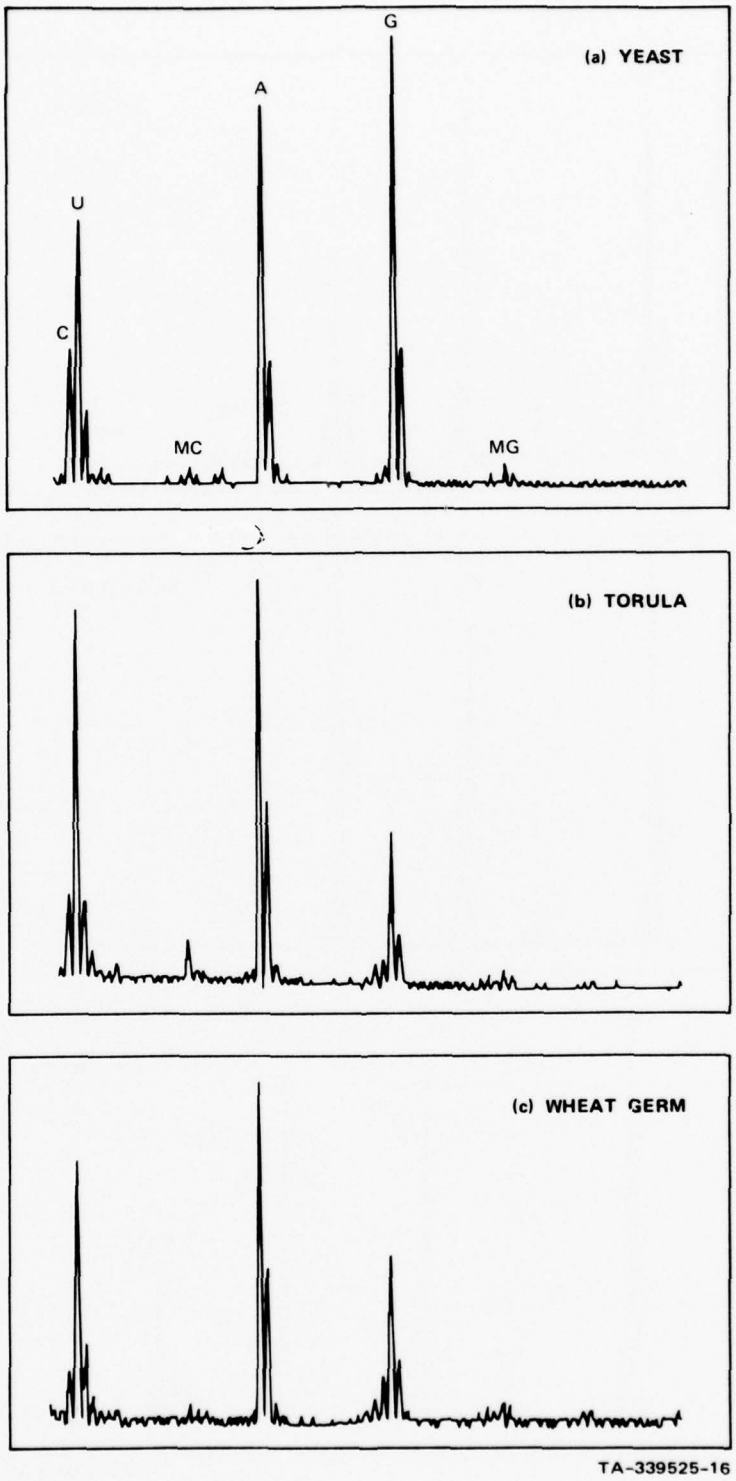


Figure 8. "Fingerprint" Pattern from High Molecular Weight RNAs

bases have been prepared. Although these derivatives increase the volatility of the parent molecule, there is no resultant change in sensitivity. All the free bases are volatile at temperatures below 300°C and yield nonfragmented field ionization spectra containing only the molecular ions of the base and its protonated form. The relative intensities of these two ionic forms are constant for samples that have been prepared and evaporated in the same way. Cytosine shows the largest relative intensity of the protonated form.

Although guanine vaporizes without decomposition, it tends to be strongly adsorbed to surfaces, even at elevated temperatures. The silanization of the ion source minimizes losses of the sample following evaporation and reduces the problems of sample memory effects. Also, the short sample path length of the new rapid-temperature-cycle source minimizes adsorption. However, the high surface activity of this compound results in a low yield when it is present in smaller quantities, and often it is almost totally adsorbed on surfaces of the sample holder and ion source. In multiple analyses of samples, we have observed that for several runs, the levels of guanine will be abnormally low, followed by a number of runs where the levels of guanine will be abnormally high. We attribute this behavior to the uncontrolled adsorption and desorption of guanine from surfaces in the source.

7-Methylguanine volatilizes at a lower temperature than guanine and adsorbs to the same sites in the source as guanine does. Hence, when no methylguanine is expected naturally in the sample, the addition of 7-methylguanine to the sample allows guanine to be efficiently transported through the source as it is evaporated from the sample holder. The use of 7-methylguanine as a carrier does not, however, appear to solve the problem of the adsorption of guanine upon some as yet undetermined component of the hydrolysate, thus lowering its ultimate yield.

We also analyzed the corresponding nucleosides by FIMS. The nucleosides show slightly more complex spectra, containing ions of the base and the sugar in addition to the molecular ions. Guanine nucleosides are the exception, yielding only $M-H_2O$ ions rather than M as the largest entity. This behavior of the nucleosides is most likely caused by thermal decomposition and not by ionization-induced fragmentation, because we do not observe significant differences in the spectra under a diversity of ionization conditions.

The ionization efficiencies of the bases are comparable, and a total ion transport efficiency of 1×10^{-7} is routinely obtainable for the magnetic sector instruments. DNA contains about 10% of each of the four major bases, i.e., 10^{-11} gm of DNA contains about 10^{-14} moles or 6×10^{9} molecules. These would produce 600 detected ions that can be determined

with ($\sqrt{600}/600$) x 100 = 2% precision. Assuming that the average organism has a weight of 10^{-12} gm, the nucleic acid content of bacteria is approximately 2%, or 2 x 10^{-14} gm/cell, and assuming a 50% extraction-hydrolysis yield, 10^4 bacteria with 1.3% precision. The analysis of viruses, which have a thousand times smaller mass but contain ten times higher concentrations of nucleuc acid, would require 10^5 or 10^6 virus particles, respectively, to attain the same precision. These estimates of sensitivity and precision are based on the assumption that each base can be monitored 100% of the time. This is not achieved with repetitive multiscanning over a 60 amu range, so that larger quantities of bacteria (10^6 to 10^7 cells) have been used in our experiments. Should the information content of mucleic acid base ratios prove sufficient for identification, these estimated sensitivities could be achieved by the use of a multicollector instrument.

B. ^{15}N-Labeled *E. coli*

1. Growth and Harvest

An *E. coli* strain that grows on completely synthetic medium was grown in a medium with $(^{15}NH_4)_2SO_4$ (98% ^{15}N) as the sole source of nitrogen. The resulting bacteria, when lyophilyzed, serve as carrier for an unknown bacteria sample, as well as a labeled internal standard for each major base encountered in either DNA or RNA.

The *E. coli* strain B25, provided by Dr. E. Snell of the Biochemistry Department, University of California, Berkeley, was grown on the following medium:

0.7%	K_2HPO_4
0.3%	KH_2PO_4
0.03%	$(^{15}NH_4)_2SO_4$
0.01%	$MgSO_4 \cdot 7H_2O$
0.001%	$CaCl_2 \cdot 2H_2O$
0.05%	$FeSO_4 \cdot 7H_2O$
0.2%	Glucose

The ^{15}N labeled *E. coli* were obtained in quantity by sequential growth to the beginning of the maximum absorbance (culture turbidity) stage in increasing volumes of medium. The third sequential culture was harvested in a Sorvall RC2-B ultracentrifuge and washed twice with distilled water. The final paste was suspended in three volumes of distilled water and freeze-dried. The bacteria were stored in this state under refrigeration.

2. Use as a Multicomponent Internal Standard and Carrier

The ^{15}N-labeled DNA from the ^{15}N-labeled E. coli was isolated and hydrolyzed to its bases. The FIMS spectrum of this hydrolysate showed cytosine at m/e 114, thymine at m/e 128, adenine at m/e 140, and guanine at m/e 156, indicating complete labeling of each base nitrogen.

Thus, the addition of a quantity of the lyophilized labeled E. coli to a sample of microorganisms to be analyzed introduces a known ratio of ^{15}N-labeled bases against which the ratio of unlabeled bases of the sample can be compared. Hence, the base composition of the microorganism under study can be determined, even though a certain base, e.g., guanine, does not ionize with the same efficiency as the other bases due to adsorption in the source and low volatility.

A quantity of the lyophilyzed E. coli added to a sample provides a carrier ^{15}N-labeled nucleic acid that allows a relatively smaller quantity of unlabeled nucleic acid from an unknown microorganism to be extracted with a high efficiency.

In addition, the final hydrolysate contains a labeled internal standard for each unlabeled base. This allows quantitation of each unlabeled base and the computation of base ratios since both forms of a given base are analyzed with the same relative efficiency.

C. Extraction of Nucleic Acids from Microorganisms

1. DNA from Bacteria

Several of the known methods for DNA isolation were investigated during phase III and were evaluated from the standpoints of speed, purity of product, and quantity of starting material required. In all these experiments, B. subtilis was employed as the source of DNA. The methods of isolation have included:

(1) Phenol extraction techniques, where DNA is separated from other cellular constituents by shaking with phenol in the presence of a denaturing agent such as sodium dodecyl sulfate. The techniques of Saito and Miura,[4] Colter et al.,[5] and Midgely[6] were followed.

(2) Separation of DNA from proteins and RNA on hydroxyapatite in 8M urea. A modification of the method of Meinke et al.,[7] was used.

(3) Extraction of the DNA with hot trichloroacetic acid (TCA). The methods used are described by Fitz-James and Dennis and Wake.[8,9]

Although the phenol extraction technique appears to be most desirable from the viewpoint of purity of the product, the efficiency of extraction decreases markedly in small samples, most probably because of occlusion of the DNA in denatured protein.

The hydroxyapatite column method suffers principally from the fact that the DNA is obtained in 8M urea, from which it is difficult to separate the DNA completely. Ultrafiltration in this solvent was not found to be effective.

The TCA extraction technique is highly efficient when small samples are handled, but as conventionally performed it is rather slow, and the product obtained is quite impure.

Anionic detergents, such as sodium dodecyl sulfate (SDS), are powerful agents for releasing nucleic acids from proteins and lipoproteins, and for inhibiting the action of nucleases. We have observed that the SDS added to promote the release of the nucleic acids of E. coli seems to be completely removed from solution during a conventional deproteiniation step using 4% isoamyl alcohol/chloroform.

CTAB (cetyltrimethylammonium bromide) is a strong, long aliphatic hydrocarbon detergent that complexes to nucleic acids via interactions with the phosphate groups of the long backbone chains and precipitates the nucleic acids from an aqueous solution.[10] Thus, after centrifugation, the nucleic acids can be recovered from the upper (aqueous) layer by addition of CTAB, and no coprecipitation of CTAB-SDS occurs.

Combining these steps with classical techniques, we have developed the following procedure that satisfies our requirements for DNA extraction. The individual steps of the extraction procedure, with times required for each step, are as follows:

Step 1	Centrifuge and wash bacterial suspension.	15 minutes
Step 2	Resuspend in 1.0 ml buffer. (50 mM tris; 2mM EDTA; 0.2% SDS).*	
Step 3	Add 0.1 ml (0.1 vol) 5 M NaCl solution	
Step 4	Heat to 80°C.	2 minutes
Step 5	Sonicate suspension (25 watts).	1 minute
Step 6	Dilute 1:1 with H_2O.	

Continued . . .

*Some bacteria (e.g., Staphylococcus aureus) require a 10-minute treatment with a pronase following Step 2, in the same buffer, to aid in breaking up the protein coat around the bacteria.

Step 7	Extract/denature proteins with 1 vol 4% isoamyl alcohol in $CHCl_3$.	2 minutes
Step 8	Centrifuge	5 minutes
Step 9	Remove upper aqueous layer.	
Step 10	Add RNAse.	
Step 11	Incubate at 37°C.	10 minutes
Step 12	Add 1/2 vol 3% CTAB solution	
Step 13	Allow precipitate to form at room temperature.	10 minutes
Step 14	Add 1.0 ml Et_2O and extract the aqueous suspension.	
Step 15	Centrifuge	5 minutes
Step 16	Remove interface precipitate and blow off excess Et_2O.	
Step 17	Wash precipitate with 1.0 ml H_2O.	
Step 18	Centrifuge.	
Step 19	Wash precipitate with 90% EtOH/0.1 M NaOAc.	
Step 20	Centrifuge.	5 minutes
Step 21	Wash precipitate with absolute EtOH.	
Step 22	Centrifuge	5 minutes
Step 23	Obtain dry DNA precipitate	
	Total time for extraction:	65 minutes

With this procedure, using 10^9 bacteria cells, the yield of DNA was approximately 50% for most bacteria. Some bacteria need to be treated with pronase, as indicated above, to be effectively extracted, hence a very general scheme of DNA isolation should include the pronase step.

We have found that the DNA strands fragment under sonication whenever they are left in the very brittle double-stranded helical structure, which is the native form in bacteria. By heating the washed bacterial suspension at 80°C, the strands of DNA are denatured to single strands. These strands are not easily fragmented when the heated culture is sonicated to break up the bacteria cell. Since CTAB precipitation proceeds more effectively for the longer strands of DNA, the heating step in the isolation is important to the isolation efficiency of our procedure. Sonication at the correct temperature/buffer conditions of the isolation procedure does not seem to affect the quality of DNA isolated, for up to 1 minute of sonication at 25 watts.

The samples of bacteria used in developing the extraction procedure were suspensions in culture medium which necessitated separation of the cells from accompanying medium constituents and growth products. To adapt to the air sampling system, it may be possible to wash the bacteria from the collection tape with the buffer used in step 2, thus saving 15 minutes of processing time.

2. DNA from a Sample Labeled with ^{15}N E. coli

A 1.0 mg sample of the lyophilized ^{15}N-labeled E. coli was added to an aliquot of sample bacteria culture after the bacteria had been harvested and washed. The extraction procedure described above was used on the mixture. Representative spectra of several species of bacterial DNA, spiked with ^{15}N-labeled E. coli and hydrolyzed in liquid HFl with HCl to bases (see below), are shown in figures 9 and 10. Analyses of these spectra are described in section 11E.

Although RNA isolation and multilabeling have not been investigated in detail under phase III of this project, this alternative approach to microorganism identification has been partially developed. A scheme to isolate total RNA from bacteria is given in table 1. Since the RNA isolation procedure differs from the DNA isolation only after step 15, both DNA and RNA can be isolated from the same sample and can be directly compared with each other for more definitive identifications when necessary.

D. Rapid Hydrolysis of DNA and RNA Using LHF and HCl

After the nucleic acids have been isolated from the microorganisms, they must be broken down into their component bases for analysis. Rapid, complete, and nondegradative hydrolysis of the nucleic acids was the subject of study under phase II of this project.

The hydrolysis systems most often reported for nucleic acid hydrolysis include 6 N HCl, 12 N $HClO_4$, or similar strong mineral acids. Hydrolysis times of up to 12 hours at room temperature or 1-2 hours at 100-200°C have been reported. We investigated several of these older methods where the reported products of these systems were analyzed by paper or column chromatography combined with uv spectroscopy. Our results verified the reported requirements for long hydrolysis times, and we were able to follow the early liberation of purines and their accelerated decomposition and the hydrolyses times and temperatures required to release the pyrimidines. An added disadvantage of these methods is the relatively large volume of aqueous solvent that must be removed before analysis.

A system based on 50% formic acid with 50% trifluoroacetic acid had similar kinetic properties and time-temperature requirements. With this system, fair yields could be obtained in 45-60 minutes at 200°C with significant losses of purines, although the volatility of the acids allowed a rapid recovery of the hydrolysis products.

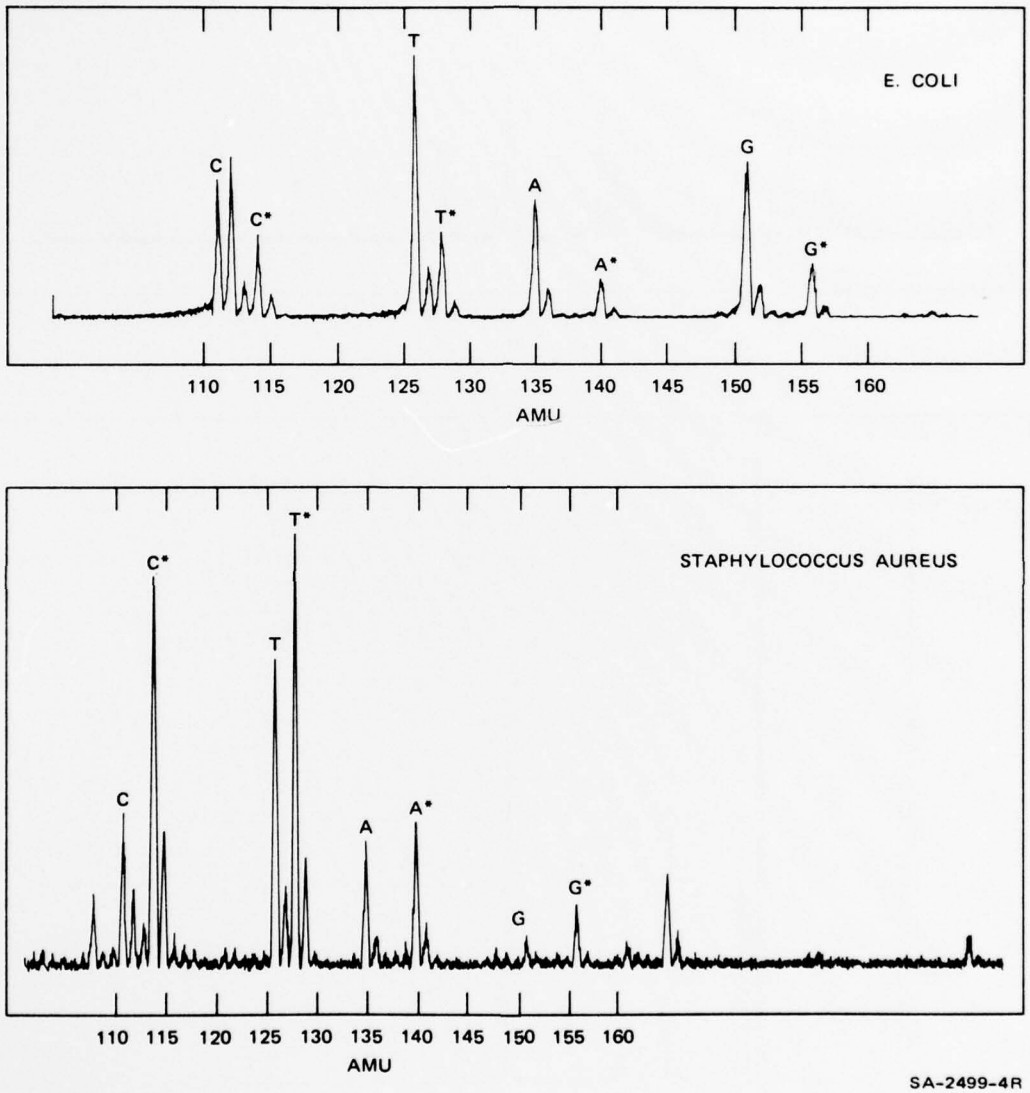
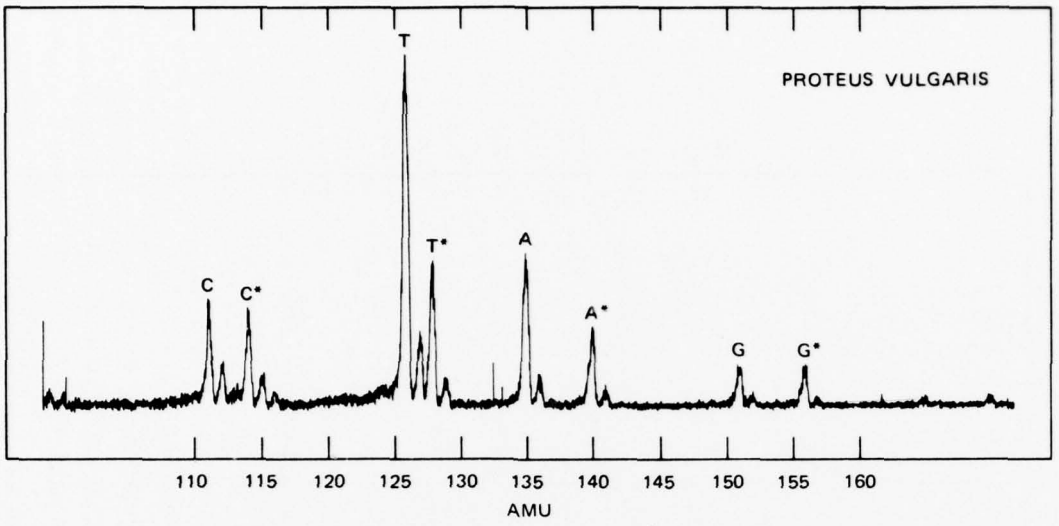


Figure 9. DNA Hydrolyzed from Bacteria Labeled with ^{15}N E. coli



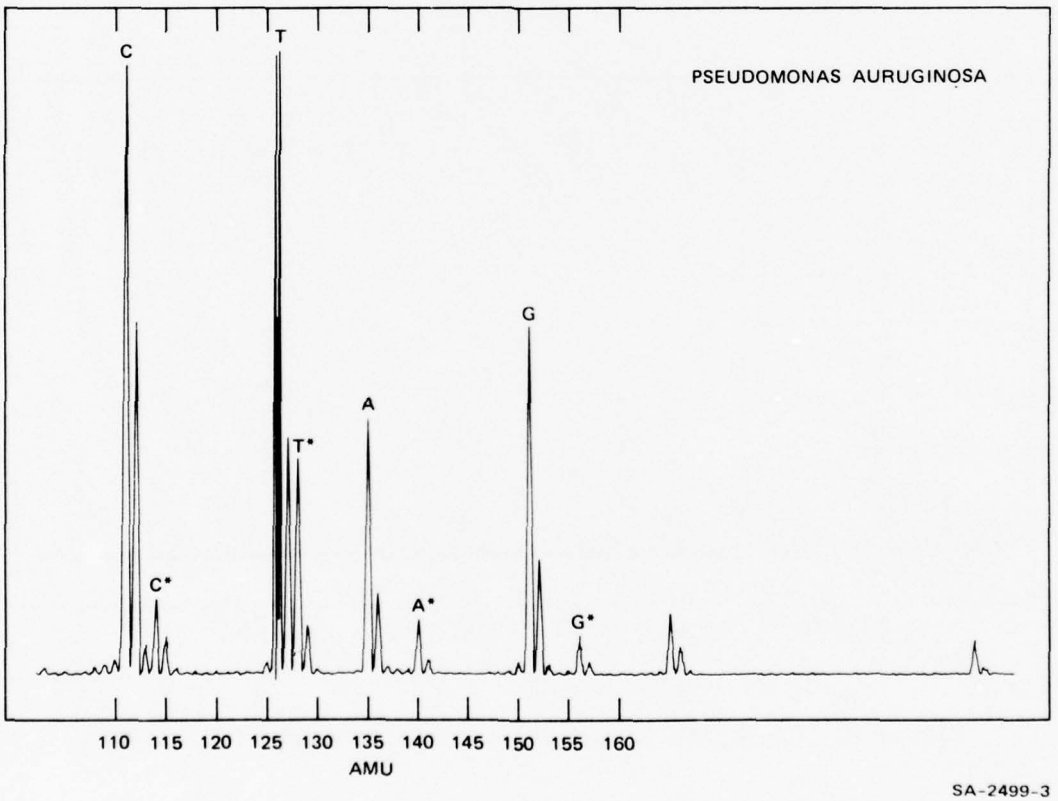


Figure 10. DNA Hydrolyzed from Bacteria Labeled with ^{15}N *E. coli*

Table 1.

Procedure for RNA Isolation

(Steps 1 - 14 are Identical for Both DNA and RNA Extractions)

Step	DNA Extraction	Time	RNA extraction	Time
		minutes		minutes
15	Centrifuge	5	Centrifuge	5
16	Remove interface precipitate		Remove lower (H_2O) layer	
17	Wash precipitate with 1 ml H_2O		Extract with 4% Isoamyl alcohol in $CHCl_3$	
18	Centrifuge	5	Centrifuge	10
19	Wash precipitate with 90% EtOH 0.1 M NaOAC		Remove upper (H_2O) layer	
20	Centrifuge	5	Dry down H_2O at 80°C	10
21	Wash precipitate with absorbent EtOH		Wash precipitate with 90% EtOH/0.1 M NaOAc	
22	Centrifuge	5	Centrifuge	5
23	Obtain DNA precipitate		Wash precipitate with absolute EtOH	1
24			Centrifuge	5
25			Obtain RNA precipitate	
		Total time 65		Total time 80

Recently, the use of anhydrous liquid hydrofluoric acid (LHF) for nucleic acid hydrolysis at room temperature without base decomposition has been reported.[10,11] Despite the fact that purines appeared to be labile in any hydrolysis system capable of liberating pyrimidines, the occurrence of LHF hydrolysis at room temperature in 60-80 minutes indicated that a faster hydrolysis might be obtained if an appropriate catalyst were used. Trifluoroacetic acid, BF_3, and HCl were investigated as catalysts, of which the latter proved to be by far the most effective.

We have shown that DNA can be effectively hydrolyzed into its bases with LHF and a catalytic amount of pure HCl at room temperature in less than 10 minutes. Using the background work of Lipkin[11] on nucleotide hydrolysis and the apparatus and techniques of Sakakibara,[12] we hydrolyzed calf thymus DNA in LHF by transferring the HF gas with liquid nitrogen in a vacuum line made of Daiflon*. The hydrolysis was followed by gas chromatography after quantitative derivatization of the bases with BSTFA reagent [Bis-(trimethylsilyl)-trifluoracetamide].

The HCl-catalyzed LHF hydrolysis of DNA is far superior to the traditional methods of hydrolysis. The very rapid hydrolysis of DNA at only 25°C provides an extremely stable environment for the bases once they are isolated from the DNA molecule. Thus, a true evaluation of base percentages in genetic DNA is possible in a very short time.

DNA hydrolysis was virtually complete in 8 minutes when a catalytic amount of pure HCl was used in the reaction (approximately 5×10^{-4} mole HCl with 400 μg calf thymus DNA and 3.0 ml LHF). Figure 11 shows the results of 2, 4, and 8 minutes of hydrolysis in LHF with HCl catalyst. The persistence of an equimolar base mixture under identical conditions shows that there is no degradation of bases during the hydrolysis (see figure 12.

The bases released are isolated as free bases, not as the nucleosides or nucleotides of the bases. According to Lipkin,[10] the phosphate-ribose backbone of the DNA molecule is first fragmented by LHF into phosphorofluoridates and nucleosides. The nucleosides are then split into their constituent sugars and bases at 25°C. The HCl added to the LHF primarily enhances the splitting of the nucleosides to form the bases, although there may be some effect on the phosphate-sugar backbone degradation. In any case, the resulting mixture of bases can be much more accurately analyzed than the mixture of bases and nucleosides obtained from other techniques.

*Registered trade name of poly-trifluoromonochloroethylene by Daikin Kogyo Co., Ltd., Osaka, Japan.

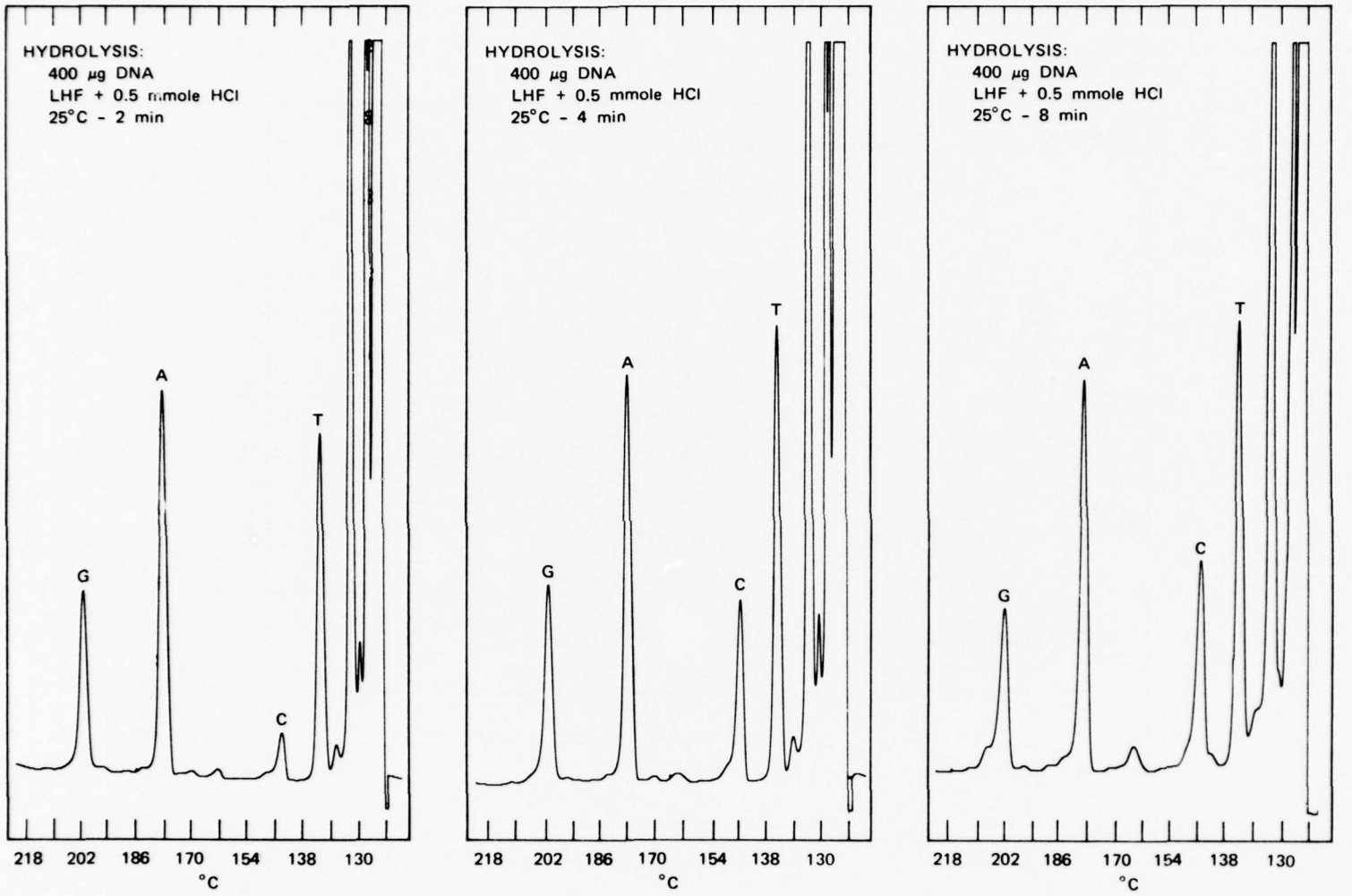


Figure 11. Gas Chromatograms of Bases Hydrolyzed from Calf Thymus DNA

In all chromatograms, 3 µl of BSTFA derivatized hydrolysate was injected into G.C. Column is 1.0 m x 4 mm glass packed with 5% OV-1 on Anakrom Q80/90. Attenuation is 1.6 x 10^{-9} amps full scale. Temperature program: 2 min at 130°C, 8°/min from 130° to 240°C.

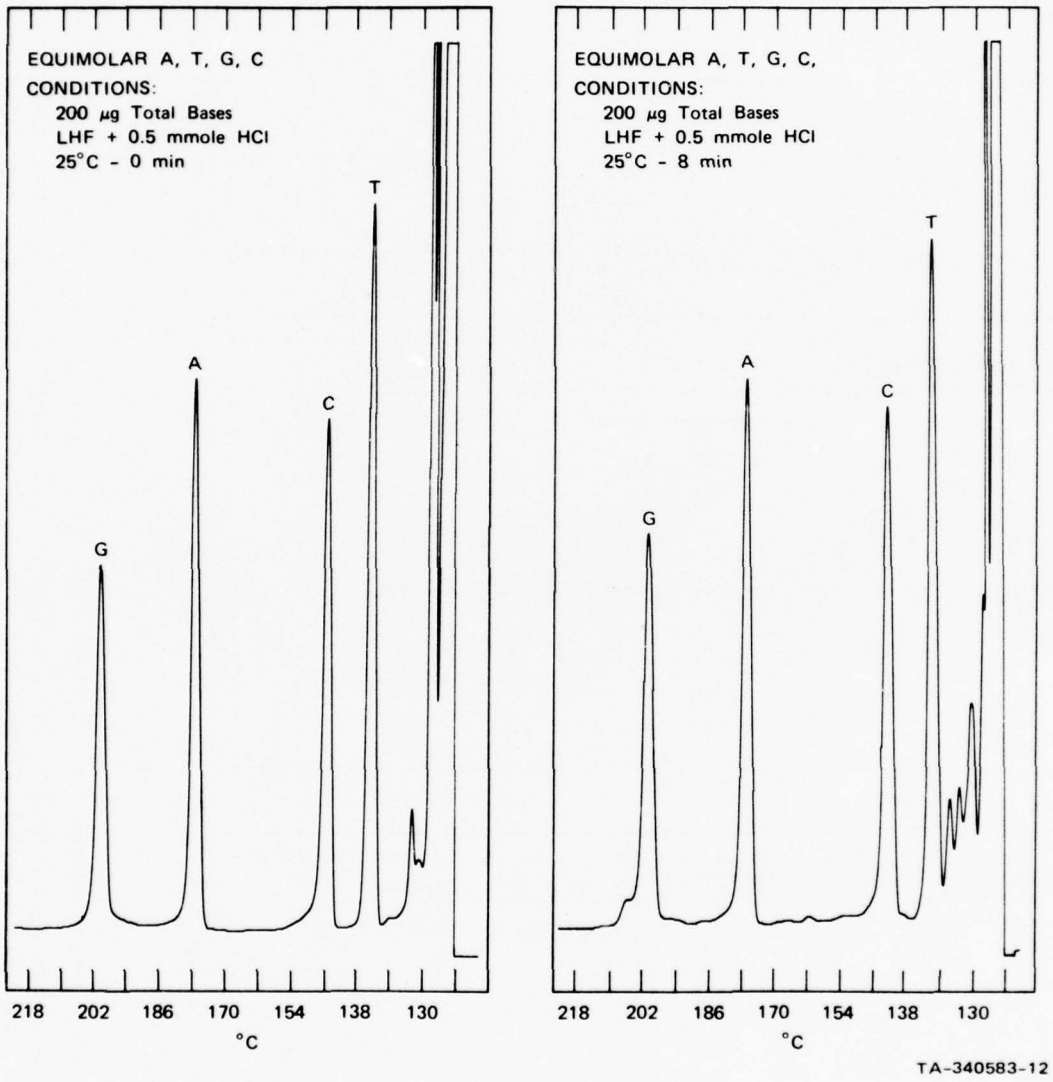


Figure 12. Gas Chromatograms of Equimolar Base Mixtures Subjected to Hydrolysis Conditions

In all cases, 3 µl of BSTFA derivatized hydrolysate was injected into G.C. Column is 1.0 m x 4 mm glass packed with 5% OV-1 on Anakrom Q80/90. Attenuation is 1.6×10^{-9} amps full scale. Temperature program: 2 min at 130°C, 8°/min from 130° to 240°C.

RNA hydrolysis is similar to the DNA hydrolysis in the LHF and HCl system. A higher temperature is required--35°C, and a longer time is needed for complete hydrolysis--16 minutes. The initial steps produce nucleosides, but these are split to free the bases very quickly in this system.

We have published a paper concerned with this hydrolysis procedure [Analytical Biochemistry 71, 519-526 (1976)]. A copy of this paper is included as an appendix to this report.

When LHF and HCl hydrolysates were analyzed using mass spectrometry, we found that guanine was apparently not properly eluted from the hydrolysate. Since hydrolysis yields guanine quantitatively, as shown by both gas and high-pressure liquid chromatography, there is an effect due to the hydrolysate matrix or the solvent.

Elution of the hydrolysate with formic acid alone or formic acid in methanol did not recover guanine but did produce a peak at m/e 177 in the mass spectrum. This peak is due to a complex formed from guanine in the presence of formic acid and some unknown component in the hydrolysate, since this peak does not appear in pure equimolar base mixtures dissolved in formic acid. The best recovery of guanine was obtained by eluting the hydrolysate with water. Although guanine is essentially insoluble in water, the dried hydrolysate is acidic enough to allow elution of guanine and other bases simply by adding water.

Further studies on the hydrolysis of DNA isolated using the technique described earlier showed that 15 minutes of hydrolysis time was needed to completely free all bases. This is probably due to the presence of proteins and other impurities that are still attached to the DNA matrix after our rapid isolation from bacteria. The original observation on the release of the bases from DNA in LHF with HCl in 8 minutes was made using very pure, long-stranded calf thymus DNA.

E. Quantitative Analysis of Bases

Earlier work on this project showed that, given a definite fixed ratio of bases, e.g., A, T, G, C, some bases would come through the field ionization mass spectrometer with varying relative efficiencies. Hence, the simple mass spectrometric analysis of a hydrolysate of DNA is not reproducible enough for microorganism identification purposes.

The addition of ^{15}N-labeled *E. coli* at the beginning of the DNA isolation procedure tends to eliminate the variability of results, since the labeled bases are analyzed with the same ionization efficiencies as the unlabeled bases at all times. During Phase III of this project, we dealt with the analysis of this labeled system.

In a dilution experiment with labeled and unlabeled bacteria, we measured ion counts: I_C, I_T, I_A, I_G (for cytosine, thymine, adenine, and guanine, respectively) and I_C^*, I_T^*, I_A^*, I_G^*, (for the labeled bases). The experimentally observed ion current for any base may be expressed as:

$$I_B = Q \cdot N_B \cdot Y_B \quad ,$$

where Q is the quantity of bacteria containing base B processed in the experiment, N_B is the fraction of base B in the DNA, and Y_B is the total experimental yield of base B including extraction, hydrolysis, solubility, volatility, and ionization. In general, Y_B is different for each base and may also vary from one experiment to the next for the same base. Taking any base and its labeled analog:

$$I_A = Q \cdot N_A \cdot Y_A$$

and

$$I_A^* = Q^* \cdot N_A^* \cdot Y_A^*$$

Since A and A^* are chemically identical, their yields will be identical, or $Y_A = Y_A^*$.

Therefore,

$$I_A / I_A^* = \frac{Q}{Q^*} \cdot \frac{N_A}{N_A^*} \quad .$$

Similarly,

$$I_T / I_T^* = \frac{Q}{Q^*} \cdot \frac{N_T}{N_T^*} \quad .$$

Thus the ion current ratio of a base to its labeled analog equals the ratio of concentrations of the base in the DNAs of the two bacteria times the ratio of the quantities of the two bacteria.

If we consider any pair of bases:

$$\frac{I_A}{I_A^*} : \frac{I_T}{I_T^*} = \frac{Q}{Q^*} \cdot \frac{N_A}{N_A^*} : \frac{Q}{Q^*} \cdot \frac{N_T}{N_T^*} = \frac{N_A}{N_T} \cdot \frac{N_T^*}{N_A^*}$$

Thus an experiment yields six ratios of this type. These ratios are assumed to be independent of both the relative yields of each base and the relative quantities of the two bacteria.

For simplicity, we define

$$[A:T] = \frac{I_A}{I_T} \cdot \frac{I_T^*}{I_A^*} = \frac{N_A}{N_T} \cdot \frac{N_T^*}{N_A^*} \quad .$$

If the base ratios of the ^{15}N-labeled bacteria are known to sufficient accuracy, the base concentrations of the unlabeled bacteria may be determined as follows:

$$\frac{N_A}{N_T} = \frac{I_A}{I_T} \cdot \frac{I_T^*}{I_A^*} \cdot \frac{N_A^*}{N_T^*} \quad ,$$

since the base ratios such as N_A^*/N_T^* are known.

Since accurate base ratios are not known, it is better at this stage to simply measure the composite ratios defined above. If unlabeled E. coli B25 is analyzed with the ^{15}N E. coli of the same strain, then all ratios should equal unity, while a different unlabeled bacteria should give ratios that differ from unity.

The six composite ratios provide six independent variables only in the case of single-stranded viral DNA. For the more common double-stranded DNA species it can be shown that only one independent variable is obtained. First, Chargaff's rule provides two equations since the formation of A-T and G-C base pairs between the strands requires that $N_A = N_T$ and $N_C = N_G$ for the total double-stranded genome. This means that the ratios $[C:G]$ and $[T:A] = 1$ for any experiment using double-stranded DNA. A third equation relating the mole fractions of the four bases is provided by the fact that their sum must equal one. These three equations plus the measurement of a single mole fraction serve to determine the six ratios. The presence of minor bases has not been considered in this analysis; however, their contribution would not significantly alter the conclusion that DNA analysis above does not provide sufficient information for a general identification procedure.

In spite of the above limitations, we have performed DNA analyses of several different bacterial strains spiked with ^{15}N-labeled E. coli Since the six ratios are internally consistent rather than unique, they allow an accurate assessment of the validity of our assumptions regard ing

the use of the labeled standard DNA. The ratios obtained in replicate experiments also show the variation to be expected and the dependence of the ratios on criteria such as the relative strength of the cell walls of different organisms.

Table 2 shows our results reported in this fashion, retaining [T:A] and [C:G], although they should equal 1 for double-stranded DNA. Each type of bacteria was analyzed at two or more different dilutions with ^{15}N-labeled E. coli, and the numbers are averages of all determinations.

In some cases, an experiment did not yield sufficient guanine counts to compute ratios for that base, and consequently, these figures are single determinations.

In table 2, we show that the ratios [T:A] and [C:G] do not equal unity, as predicted by Chargaff's rules. The use of the higher-sensitivity magnetic sector mass spectrometers has shown the [T:A] ratio to approach unity more closely, with a higher signal-to-noise ratio. The [C:G] ratio, however, is still dependent upon the quantities of guanine that are ionized. The addition of 7-methylguanine to the sample as a carrier aids in flushing guanine out of the mass spectrometer, but analysis of guanine is still a problem area.

Table 2.

BASE RATIO PAIRS FOR BACTERIA
COMPARED WITH ^{15}N-LABELED E. COLI

Bacteria	Base Ratio Pairs[a]					
	[C:T]	[C:A]	[C:G]	[T:A]	[T:G]	[A:G]
E. coli	1.06	1.05	0.98	0.99	0.93	0.94
Staphylococcus aureus	0.57	0.51	0.79	0.90	1.39	1.55
Proteus vulgaris	0.51	0.64	1.21	1.26	2.39	1.90
Pseudomonas aeruginosa	1.92	1.73	0.92	0.90	0.48	0.53

$$^{a}[C:T] = \frac{I_C}{I_T} \cdot \frac{I_T^*}{I_C^*} = \frac{N_C}{N_T} \cdot \frac{N_T^*}{N_C^*}$$

III DISCUSSION AND RECOMMENDATIONS

In spite of the recurring problems of guanine analysis and the limited information content of double-stranded DNA base ratios, the use of ^{15}N-labeled DNA has allowed us to establish the feasibility and desirability of multicomponent isotope dilution for problems of this type. The techniques we have developed may easily be applied to the accurate analysis of multicomponent mixtures having more information content. The labeled bacteria also contain ^{15}N-labeled RNA that can be analyzed in place of or in addition to DNA.

With our more advanced mass spectrometric instrumentation, including field desorption, it may be possible to analyze short oligonucleotide sequences from either DNA or RNA. The amount of information obtained from multicomponent isotope dilution experiments with DNA should allow us to refine and improve our sample processing methodologies to obtain better accuracy and reproducibility.

The extraction procedure developed for DNA can, with only slight modification, be used to recover both RNA and DNA for analysis. This procedure, given in table 1, differs from the DNA extraction procedure only beyond step 15.

The analysis of RNA may provide more information than DNA analysis although some of the variation in RNA composition may be sensitive to environmental or growth conditions as well as to species differences. An investigation of the optimum sample for identification purposes, whether DNA, RNA, total nucleic acids, or base sequences will be the subject of investigation under our latest contract.

LITERATURE CITED

1. E. Chargaff and J. N. Davidson, Eds., The Nucleic Acids (Academic Press, New York, 1955), Vol. I, p. 307; Vol. III, p. 147; Vol III, p. 295.

2. E. Borek and R. S. Srinivasan, Ann. Rev. Biochem. 35, 275 (1966).

3. H. L. Brown, R. H. Cross and M. Anbar, Characterization of Multipoint Field Ionization Sources, Int. J. Mass Spectrom. Ion Phys, in press (1977).

4. H. Saito and K. Miura, Preparation of Transforming Deoxyribonucleic Acid by Phenol Treatment, Biochim. Biophys. Acta 72, 619 (1963).

5. J. S. Colter, R. A. Brown, and K.A.O. Ellem, Observations on the Use of Phenol for the Isolation of Deoxyribonucleic Acid, Biochim. Biophys. Acta 55, 31 (1962).

6. J.E.M. Midgely, Hybridization of Microbial RNA and DNA, Methods Microbiol. 5A, 331 (1971).

7. W. Meinke, D. A. Goldstein, and M. R. Hall, Rapid Isolation of Moose DNA from Cells in Tissue Culture, Anal. Biochem. 58, 82 (1974).

8. P. C. Fitz-James, The Phosphorous Fractions of Bacillus cereus and Megaterium, J. Microbiol. 1, 502 (1955).

9. E. S. Dennis and R. G. Wake, Autoradiography of the Bacillus subtilis Chromosome, J. Molec. Biol. 15, 435 (1966).

10. H. Morimoto et al., Spectrophotometric Analysis of RNA and DNA Using Cetyltrimethylammonium Bromide, Anal. Biochem. 62, 436 (1974).

11. D. Lipkin, B. Phillips, and J. A. Brell, The Action of Hydrogen Fluoride on Nucleotides and Other Esters of Phosphorus Acids, J. Organ. Chem. 34, 6 (1969).

12. D. Sakakibara, Y. Shimonshi, Y. Kishida, et al., Use of Anhydrous Hydrogen Fluoride in Peptide Synthesis, Bull. Chem. Soc. Jpn. 40, 9 (1967).

APPENDIX

ANALYTICAL BIOCHEMISTRY 71, 519–526 (1976)

Acid-Catalyzed Release of Purines and Pyrimidines from Nucleic Acids in Liquid Hydrogen Fluoride

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The quantitative release of purines and pyrimidines from DNA is accomplished in anhydrous hydrogen fluoride containing catalytic amounts of hydrochloric acid within 12 min at 25°C. The release of bases from RNA is slower, requiring 20 min at 35°C. No degradation of the major bases takes place under these conditions, although 5-hydroxymethyluracil is decomposed. The methodology offers a significant improvement in terms of both time and cleanliness of product over conventional solvolytic methods for the degradation of nucleic acids to free bases.

For the mass spectrometric or gas chromatographic analysis of nucleic acid bases, it is convenient to use acids that not only can degrade the nucleic acids quantitatively to free bases, but that also can be removed completely from the reacted samples by evaporation. Trifluoroacetic acid, 88% formic acid, and mixtures of the two are generally satisfactory catalysts when reaction is carried out under strict exclusion of air at 175°C for 1 to 2 hr (1–3). A more rapid and less destructive method of solvolysis, however, would be useful in clinical or biochemical laboratories seeking to ascertain the base compositions of uncharacterized nucleic acid preparations or to recover quantitatively a given base incorporated into DNA or RNA.

Kanaoka *et al.* (4) recently reported that liquid hydrogen fluoride (LHF) catalyzes the solvolyses of DNA and RNA to their constituent bases at room temperature in approximately 2 hr. Preliminary experiments in our laboratory indicated that gas–liquid chromatograms (glc) appeared much cleaner after LHF solvolysis of DNA at room temperature than they do after solvolysis in trifluoroacetic acid–formic acid mixtures at 175°C. These results suggested that the use of LHF at room temperature results in less product decomposition than occurs in solvolyses at high temperatures, and we were encouraged by these results to carry out an extensive investigation of the solvolyses of DNA and RNA by LHF in the presence of a stronger acid catalyst. Our goal was to decrease the reaction time to make the method useful for routine mass spectrometric or gas chromatographic analyses in the clinical laboratory.

MATERIALS AND METHODS

The DNA and RNA samples and the purine and pyrimidine bases were purchased from Calbiochem and Sigma. Anhydrous hydrogen fluoride was obtained from Air Products.

A type I HF-reaction apparatus constructed of Daiflon polymer (polytrifluoromonochloroethylene) was purchased from the Protein Research Foundation, 476 Ina, Minoh-shi, Osaka, Japan. Gas–liquid chromatographic analyses were performed on a Hewlett-Packard 5700A gas chromatograph at 1.6×10^{-9} A full-scale attenuation. The column was 4 mm × 1 m glass, packed with 5% Analabs OV-1 on Anakrom 80/90.

Solvolyses of DNA and RNA. Samples of DNA or RNA in 0.0014 M phosphate buffer solution (pH 6.8) containing 0.4 mg total DNA or RNA were placed in the Daiflon reaction vessels and dried in a water bath at 40°C under a stream of nitrogen. After attachment to the HF-reaction apparatus, the vessels were evacuated, and 3.0 ml LHF was condensed into the reaction vessels by use of liquid nitrogen.

The gaseous HCl was introduced into the apparatus until the pressure had risen by about 10 mm Hg (about 0.12 mmol), as indicated by a mercury manometer. The gas was then condensed into each vessel with liquid nitrogen. (The precise ratio of HCl to LHF is not critical; varying the HCl content from 0.04 to 0.4 mmol produced similar results.) We arbitrarily chose a starting time for all reactions of 2 min after the LHF had melted completely following immersion in a water bath maintained at a given temperature.

The reactions were stopped by immersing the vessels in liquid nitrogen for 30 sec followed by rapid (<0.5 min) removal of the gaseous HCl under vacuum at 25°C. The LHF was then distilled at 25°C into a cold trap (~10 min).

Stability of purines and pyrimidines in LHF. The stability of the bases under the reaction conditions was studied by using an equimolar mixture of adenine, thymine, cytosine, guanine, and uracil (A,T,C,G,U). The bases were dissolved in 88% HCOOH, and aliquots containing 0.2 mg of total bases were transferred to the Daiflon vessels and dried under a stream of nitrogen in the usual manner. The free bases were exposed to LHF and the hydrochloric acid catalyst for varying times by the above procedures.

Gas-chromatographic analyses. The reaction products were transferred with 88% formic acid into 1.0 ml Pierce "Reacti-vials" with two washings, and the solvent was evaporated in a 40°C water bath under a stream of pure nitrogen gas. Derivatization was carried out on the residue dissolved in 50 μl acetonitrile and 50 μl bis(trimethylsilyl)trifluoroacetamide (BSTFA) reagent in a 150°C sand bath for 30 min (3).

The derivatized base mixtures were cooled, and 3 μl of a DNA/RNA reaction mixture or 1 μl of an equimolar mixture was injected into the

Appendix

gas chromatograph. The output of the glc was calibrated by using a derivatized equimolar (A,T,C,G,U) mixture. Base ratios were calculated from the areas of the peaks as measured with a planimeter.

Thin-layer chromatographic analyses. As a check on the glc determinations, purine and pyrimidine yields were also obtained by using two-dimensional thin-layer chromatography (tlc) on Macherey–Nagel 300 HR

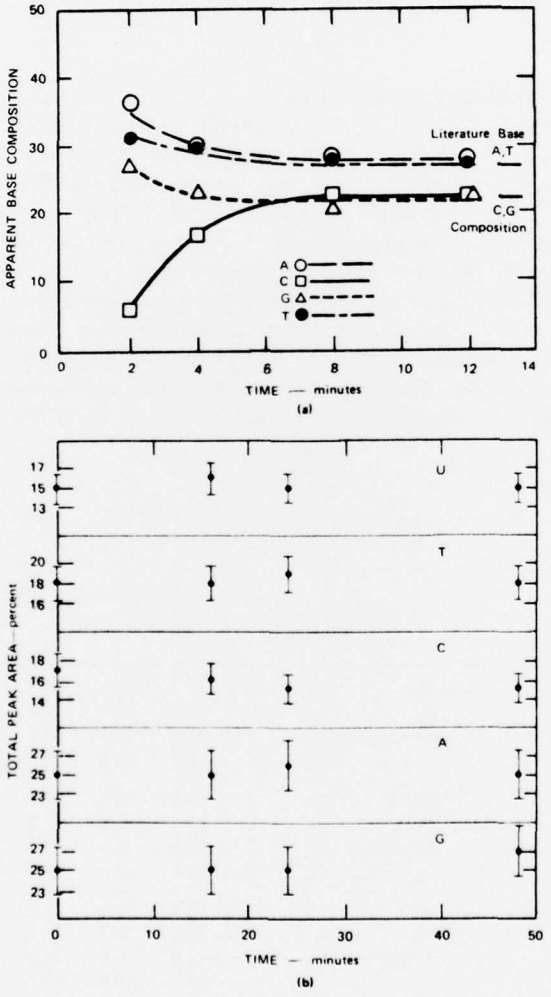

FIG. 1. (a) Base percentages resulting from solvolysis of calf-thymus DNA in LHF–HCl at 25°C. (b) Peak areas of gas–liquid chromatogram from an equimolar mixture of bases (A,T,C,G,U) subjected to LHF–HCl at 25°C for varying times.

Appendix

(high purity) cellulose, further purified by washing in the chromatographic solvents. The solvents used were: (i) isopropanol/HCl/H_2O (65/16.7/18.3); and (ii) isopropanol/H_2O/NH_4OH (75/15/1.3) (5). After the spots were located under uv light, they were scraped off and eluted four times each with 1.0 ml of 0.05 M HCl. The solutions were evaporated and redissolved in 1.0 ml of 0.05 M HCl, and the concentrations were determined spectrophotometrically (6). Blank readings were made by using scrapings from a plate that had not been spotted with a nucleic acid solution.

RESULTS

In the first phase of our investigation, we studied the solvolysis of calf-thymus DNA in LHF at 25°C. In general agreement with the findings of Kanaoka *et al.* (4), the bases were maximally liberated within 80–120 min reaction time. A quantitative assessment of the stability of the bases in LHF by use of a standard equimolar mixture of the five bases (A,T,C, G,U) revealed that no degradation of any base is observable in 4 hr. Kanaoka *et al.* (4) have already reported that the major purine and pyrimidine bases are stable in this medium overnight at room temperature. They noted, however, that the acid-labile minor base, 5-hydroxymethyluracil, is degraded by LHF in 2 hr to another compound.

To accelerate the solvolysis process, we added anhydrous HCl to the LHF reaction medium. This reagent proved to be a highly efficient

TABLE 1

MOLAR BASE PERCENTAGE COMPOSITIONS OF DIFFERENT DNA'S AFTER SOLVOLYSIS IN LHF–HCl FOR 8 MIN AT 25°C

DNA source	Analytical method	Molar base percentage composition				
		G	A	C	5MC	T
Calf thymus	glc	16.2	28.1	25.7	1.0	29.0
	tlc	22.7	28.9	22.8	—[a]	25.6
	Literature (7)	21.6	27.8	21.9	0.9	28.2
		±0.6	±0.6	±0.8	±0.6	±0.6
Chicken blood	glc	19.8	26.8	21.6	0.0	31.8
	tlc	21.1	28.8	21.6	—[a]	28.5
	Literature (7)	21.2	28.2	21.5	0.0	29.0
		±0.8	±0.5	±0.2		±0.5
Salmon sperm	glc	19.0	25.9	23.7	0.6	30.8
	tlc	22.3	28.5	22.4	—[a]	26.8
	Literature (7)	21.7	28.6	21.2	0.0	28.5
		±0.7	±0.9	±0.8		±1.5

[a] 5-Methylcytosine was not considered when calculating molar base percentage compositions.

Appendix

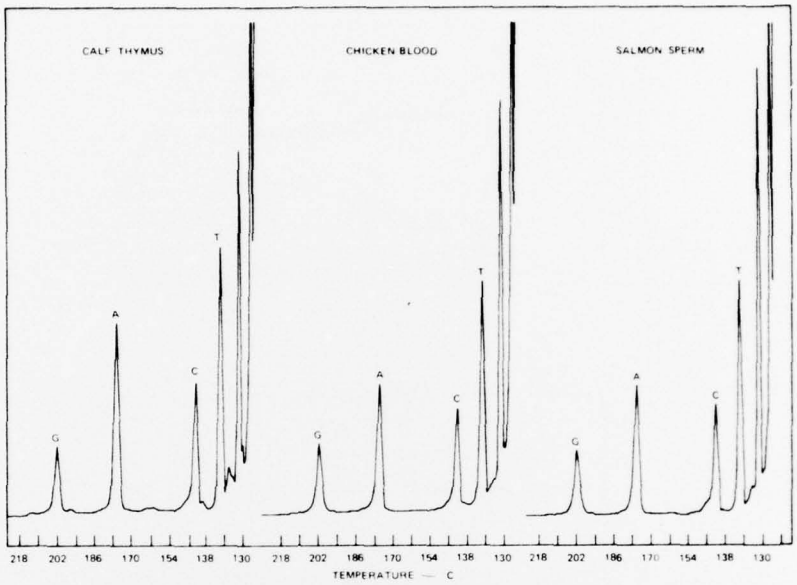

FIG. 2. Gas–liquid chromatograms of bases released from DNA's of different sources in LHF–HCl at 25°C for 8 min. Temperature program: 2 min at 130°C, 8°/min from 130° to 240°C.

catalyst. Results of a series of solvolyses of calf-thymus DNA at 25°C are shown in Fig. 1a. Solvolysis is completed in less than 12 min. After subjecting an equimolar mixture of bases (A,T,C,G,U) to LHF with the same catalytic amount of anhydrous HCL, we found no degradation of bases in 48 min at 25°C, as shown in Fig. 1b.

The ordinate of Fig. 1a is the *apparent* percentage base composition, that is, it expresses the percentage of each base relative to the total *liberated* bases at any given time. Because the bases are released at different rates, some curves will fall while others rise. This does not imply, however, that degradation is occurring. The stability of the products in the reaction medium is shown by Fig. 1b and by the fact that the apparent percentage base compositions approach their expected literature values within the experimental error.

Solvolyses of DNA from three different sources in LHF with HCl as catalyst for 8 min at 25°C gave the base compositions shown in Table 1. The base ratios calculated from two-dimensional thin-layer chromatograms are in quantitative agreement with literature values (7). The results obtained using glc (Fig. 2) are in less satisfactory agreement. Others have reported similar inaccuracies using the glc method (3), due perhaps to difficulties in obtaining quantitative derivatizations of the bases or of precisely

Appendix

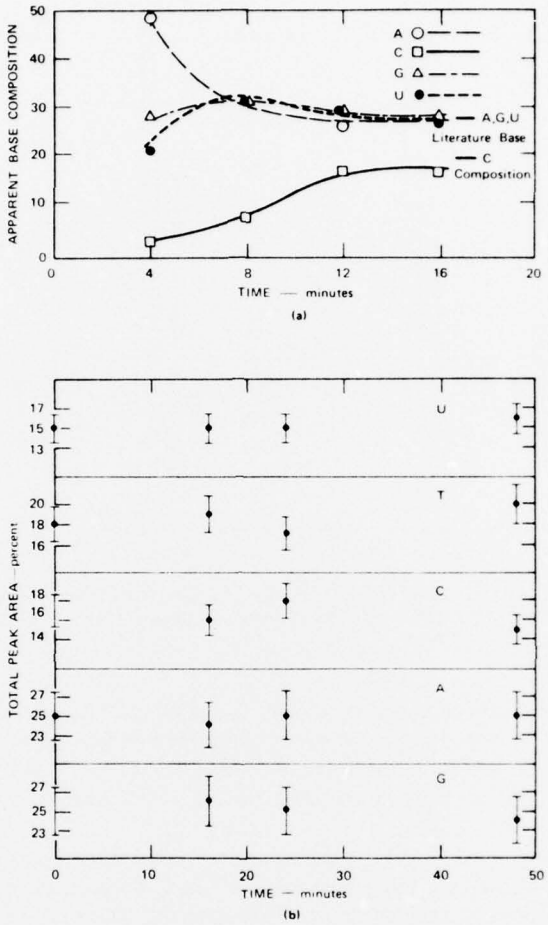

FIG. 3. (a) Base percentages resulting from solvolysis of high molecular weight yeast RNA in LHF–HCl at 35°C. (b) Peak areas of gas–liquid chromatograms from an equimolar mixture of bases (A,T,C,G,U) subjected to LHF–HCl at 35°C for varying times.

integrating chromatographic peak areas. In the present study, glc was used primarily to follow the progress of reaction in LHF–HCl and to characterize the reaction products.

Highly polymerized yeast RNA, when subjected to LHF with HCl catalyst, gives results different from DNA. At 25°C, cytosine appears not to be quantitatively released under these conditions for up to 16 min, whereas adenine seems to be completely released in 4 min. However, when the reaction temperature is raised to 35°C, cytosine is released

Appendix

TABLE 2

MOLAR BASE PERCENTAGE COMPOSITION OF HIGH MOLECULAR WEIGHT YEAST RNA AFTER SOLVOLYSIS IN LHF–HCl FOR 16 MIN AT 35°C

Analytical method	Molar base percentage composition			
	G	A	C	U
glc	28.9	27.9	17.8	25.7
tlc	28.9	26.5	19.9	24.7
Literature (9)	27.3 ±0.2	26.5 ±0.2	19.2 ±0.3	27.0 ±0.2

completely along with the other bases, as shown in Fig. 3a. Solvolysis appears to be completed in 16 min at 35°C. In contrast to the results of acid hydrolysis of RNA in aqueous media at 100°C, where pyrimidine nucleosides are formed as a final product (8), the LHF–HCl system produces free uracil and cytosine.

We examined the stability of the bases at 35°C in LHF–HCl. An equimolar mixture of bases, when treated with the solvolysis reaction medium at 35°C, yielded no noticeable degradation for up to 48 min (Fig. 3b). When we examined the stability of the acid-labile minor base, 5-hydroxymethyluracil, in LHF–HCl, however, we found that this compound was decomposed extensively within the reaction time of 16–20 min at 35°C.

Base ratios of highly polymerized yeast RNA obtained from the 16 min solvolysis in LHF–HCl at 35°C are shown in Table 2. The results appear to be in satisfactory agreement with the literature (9).

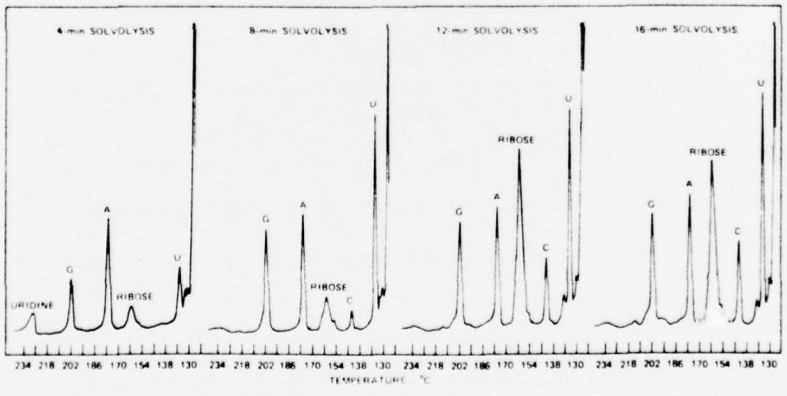

FIG. 4. Gas–liquid chromatograms of bases released from high molecular weight yeast RNA in LHF–HCl at 35°C. Temperature program: 2 min at 130°C, 8°/min from 130° to 240°C.

Appendix

DISCUSSION

The solvolysis of nucleic acids by LHF–HCl is, in general terms, similar to their solvolyses by other strong acids, but the new methodology appears to offer several significant advantages. Solvolysis of DNA at 25°C is completed in less than 12 min, and the chromatograms obtained are much cleaner and, therefore, much less subject to error than those obtained after solvolysis at high temperatures (e.g., 3). Moreover, cytosine appears as a single glc peak following LHF–HCl solvolysis. In contrast, when DNA or RNA is solvolyzed at 175°C, two peaks are ascribed to cytosine, and the ratio of the peaks is not constant (3).

The solvolysis of RNA in LHF–HCl at 35°C proceeds with the transient formation of nucleosides, and a large amount of undegraded ribose is obtained as a by-product (Fig. 4). (The identity of this peak was checked by running derivatized ribose through the glc.) Because pyrimidine nucleosides are commonly formed from RNA under acidic conditions, the formation of nucleosides was not unexpected. However, the rapid breakdown to the free bases is notable, and the chromatograms obtained are exceptionally clean. Such a method of solvolysis could, therefore, offer significant improvement over conventional methods in terms of time when searching for relatively small amounts of the minor bases (except 5-hydroxymethyluracil), or when dealing with very small quantities of nucleic acids where interferences from degradation products could become important.

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REFERENCES

1. Dutta, S. K., Jones, A. S., and Stacey, M. (1956) *J. Gen. Microbiol.* **14,** 160–166.
2. Wyatt, G. R., and Cohen, S. S. (1953) *Biochem. J.* **55,** 774–782.
3. Lakings, D. B., and Gehrke, C. W. (1972) *Clin. Chem.* **18,** 810–813.
4. Kanaoka, Y., Itoh, K., Sato, E., Nomura, A., and Mizuno, Y. (1970) *Chem. Pharm. Bull. (Tokyo)* **18,** 1475–1477.
5. Grippo, P., Iaccarino, M., Rossi, M., and Scarano, E. (1965) *Biochim. Biophys. Acta* **95,** 1–7.
6. Sober, H. A. (ed.) (1968) Handbook of Biochemistry, Section G, The Chemical Rubber Co., Cleveland.
7. Ibid., Section H.
8. Parish, J. H. (1972) Principles and Practice of Experiments with Nucleic Acids, p. 203, John Wiley, New York.
9. Fauman, M., Rabiwitz, M., and Getz, G. S. (1969) *Biochim. Biophys. Acta* **182,** 355–360.

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